# Detection of Mumps Virus Genome Directly From Clinical Samples and a Simple Method for Genetic Differentiation of the Hoshino Vaccine Strain From Wild Strains of Mumps Virus

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A simple and sensitive method was developed for the differentiation of the Hoshino vaccine strain from wild strains with a restriction fragment length polymorphism (RFLP) analysis in the part of hemagglutinin-neuraminidase (HN) gene. The virus genome was amplified by using a reverse transcriptase-polymerase chain reaction (RT-PCR) directly from clinical samples. The PCR product of the Hoshino vaccine strain was cleaved into 2 fragments after digestion with Sca I and Afl II. All wild strains showed 2 RFLP profiles, A and B, different from that of vaccine strain. Wild A strains were cut into 2 fragments after digestion with Sca I only, while wild B strains were cleaved neither with Sca I nor Afl II. This molecular approach provides an effective method for differentiation of the Hoshino vaccine strain from wild strains of mumps virus in patients after vaccination. ***J. Med. Virol. 52:195–199, 1997.*** 

**KEY WORDS:** mumps vaccine; mumps meningitis; genomic differentiation; RFLP

## INTRODUCTION

Virus isolation is the most reliable diagnostic method for virus diseases. While mumps virus isolation was first reported using human or monkey kidney cells, the isolation of mumps virus is as low as 30–40% of the samples. It has been thought that the sensitivity of Vero cells is not sufficient for isolation of mumps virus from nasopharyngeal swabs (NPS) or cerebrospinal fluid (CSF). Thus mumps is diagnosed clinically or serologically. Recently, nucleic acid amplification using the polymerase chain reaction (PCR) has been introduced for the detection of low concentration of fastidious pathogens in cell cultures [Boriskin et al., 1993]. A simple and sensitive method has been described for the detection of measles virus genome [Mori, 1994; Nakayama et al., 1995], amplifing the regions encoding the nucleocapsid (N) protein and hemagglutinin (H) protein of measles virus by a reverse transcriptase-polymerase chain reaction (RT-PCR). The AIK-C measles vaccine strain was also differentiated from the wild measles strains by restriction fragment length polymorphism (RFLP).

In the United States, usage of mumps vaccine, combined with measles and rubella vaccine (MMR), decreased markedly the number of mumps patients. MMR vaccine was introduced in Japan in 1989 for infants of 1 year of age, but combined usage for routine immunization practice was discontinued in 1993 because of the unexpectedly high incidence of aseptic meningitis [Sugiura et al., 1991]. A reliable and sensitive method was required for the differentiation of mumps virus in patients with aseptic meningitis after vaccination. Several authors reported that the Urabe strain or the Jeryl Lynn strain were differentiated from wild strains by nucleotides sequencing in the parts of phospho protein (P), fusion protein (F), small hydrophobic protein (SH), and hemagglutinin-neuraminidase (HN) genes [Yamada et al., 1991; Forsey et al., 1990; Künkel et al., 1995; Brown et al., 1991]. We sequenced the SH and HN regions to demonstrate the longitudinal chronological genetic variability of mumps virus strains since 1976 in comparison with the Hoshino vaccine strain (data not shown). The Hoshino vaccine strain, which was developed by Sasaki [1976], has been used widely in Japan. We described a simple and sensitive method for the detection of mumps virus genome directly from clinical samples and RFLP analysis of the PCR product in a part of the HN gene. This molecular approach provides an effective method for

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the differentiation of the Hoshino vaccine strain from wild strains of mumps virus in patients with complications after vaccination.

## MATERIALS AND METHODS

### Materials

Materials consisted of 52 nasopharyngeal swab (NPS) samples obtained from patients with parotitis, and 26 cerebrospinal fluid (CSF) samples obtained from patients with aseptic meningitis. We also examined 5 NPS samples obtained from patients with acute parotitis and 14 CSF samples obtained from patients with aseptic meningitis within 4 weeks after vaccination with the Hoshino vaccine strain. Pharyngeal swab samples were obtained by pharyngeal scraping with cotton swab and were soaked into 2 ml of minimum essential medium (MEM) supplemented with 5% calf serum (CS) and adequate antibiotics. Samples were stored at −70°C until virus isolation and RNA extraction. Total RNA was extracted from 200 ul of NPS and CSF, as reported by Chomczynski et al. [1987]. Briefly, 200 ul of lysis buffer containing solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% Nonidet P-40) was added. RNA was extracted by means of a phenol chloroform mixture and was precipitated with equal volume of isopropanol. The RNA pellet was resuspended in 20 ul of sterile distilled water, and 5 ul of total RNA was applied for RT procedure.

### Virus Isolation

0.1 ml of samples was inoculated on the monolayer of Vero cells on a 24-well tissue culture plate and passaged 3 times until viral-specific cytopathic effect (CPE) was observed. Samples with CPE were confirmed by a neutralization test using antiserum to mumps virus. Samples without CPE after 3 blind passages were considered negative for virus isolation.

### Primer Design and Restriction Map

We synthesized the sets of linker-primers for the HN region. The genomic locations and the sequences of the primers are depicted in Figure 1. Mumps genomic RNA was first converted to cDNA with HN1 positive sense primer located at the 5′-end of the HN coding region. The first PCR was done with a set of HN3 (+) and HN6 (−) primers, and a nested PCR experiment was done with a set of HN4(+) and HN7(−) primers. M13 sequencing primer alignment was attached at the 5′-end of HN7(−) primer for direct sequencing, which yielded the 541 by PCR product including the primer sequence. The restriction sites for Sca I and Afl II and the length of predicted restriction fragments are also depicted in Fig. 1.

### RT-PCR and RFLP

Viral genomic RNA was reverse-transcribed with AMV reverse transcriptase (Life Sciences Inc., St. Petersberg, FL) at 40°C for 1 hr. Five ul of viral cDNA was amplified by PCR in a total volume of 50 ul mixture as recommended by the manufactures, using 1.25 units of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Light mineral oil (Sigma Chemical Co., St. Louis, MO) was layered onto the top of the mixture, which was then subjected to a DNA thermal cycler (Program Temperature Control System PC-700, Astec Co., Ltd., Fukuoka, Japan). The first three cycles were at 92°C for 2 min, 55°C for 3 min, and 72°C for 2 min. These were followed by 30 cycles (at 93°C for 1 min, 58°C for 1 min, and 72°C for 2 min), with a final additional extention period of 5 min at 72°C. For nested PCR, 1 ul of the first PCR product was mixed in a total of 50 ul PCR reaction mixture and the samples were subjected to PCR using the same thermal cycle program. PCR products were electrophoresed through 3% agarose gel (NuSieve 3:1, FMC Bioproducts Corp., Rockland, ME) and visualized by staining with ethidium bromide. PCR products were digested with Sca I, Afl II (TaKaRa Biotechnology, Japan). Restriction fragments were also detected by electrophoresis in 3% agarose gels and were visualized with ethidium bromide. PCR products were sequenced for confirming the restriction enzyme sites by direct sequencing with ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

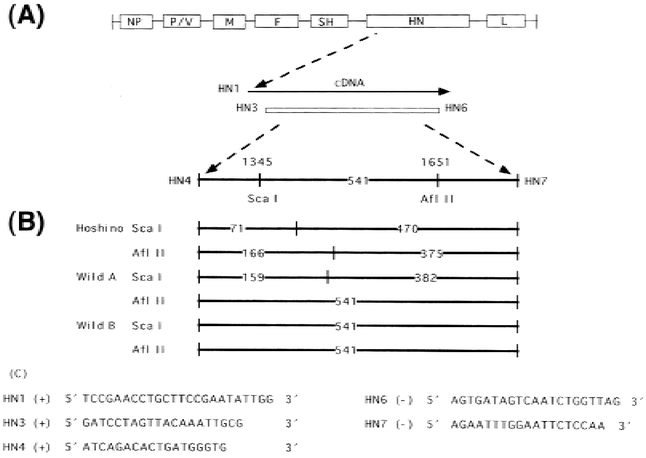


Fig. 1. Primer design and restriction site mapping. **A:** HN1(+) was used for first-stranded cDNA synthesis, HN3(+) and HN6(−) for first PCR, and HN4(+) and HN7(−) for nested PCR. **B:** the size of PCR product and predicted restriction fragments. Each number shows the fragment length predicted from above restriction map. **C:** primer sequence.

## RESULTS

### Detection of Mumps Virus Genome From Clinical Samples

Mumps virus genome was sought in 52 NPS clinical samples by using RT-PCR. Some of the results of virus genome detection and virus isolation are shown in Figure 2. Panel B shows the result of RT-PCR for NPS samples obtained from patients with acute parotitis suspected of natural mumps infection. Among 52 patients with acute parotitis, 8 patients had a past history of immunization with mumps vaccine. The results of RT-PCR and virus isolation of these 8 patients are

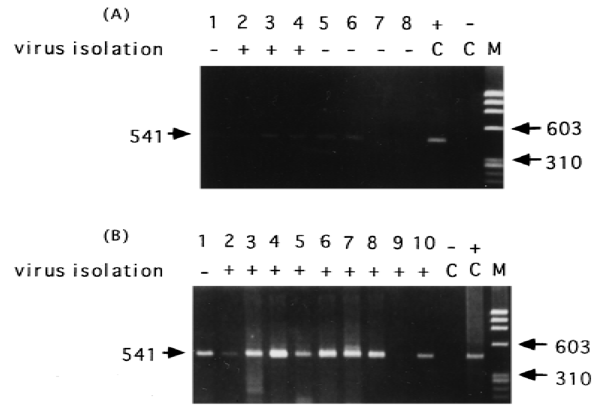


Fig. 2. RT-PCR and virus isolation from NPS obtained from acute parotitis. **A:** Detection of mumps virus genome from patients with acute parotitis who had been immunized with mumps vaccine. **B:** Detection of mumps virus genome from patients with parotitis without history of vaccination. +C, positive control; –C, negative control; M, DNA marker.

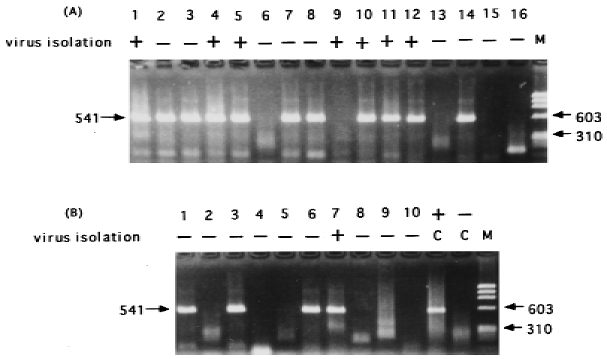


Fig. 3. RT-PCR and virus isolation from CSF obtained from aseptic meningitis. **A:** detection of mumps virus genome from patients with meningitis after MMR vaccination. **B:** Detection of mumps virus genome from patients with aseptic meningitis. CSF samples were obtained from mumps meningitis (lanes 1 to 7), febrile convulsion (lane 8), and Echo type 30 aseptic meningitis (lanes 9 and 10). +C, positive control; –C, negative control; M, DNA marker.

shown in panel A. Three out of 8 samples were positive for virus isolation, and mumps virus genome was detected in 6 samples. Despite the lack of virus isolation, some clinical samples were positive for mumps virus genome. We examined for mumps genome detection in 26 CSF clinical samples by RT-PCR. The results of detection of mumps virus genome are shown in Figure 3. Panel A shows the results of RT-PCR and virus isolation from CSF obtained from the patients with aseptic meningitis after vaccination with MMR. Mumps virus was isolated in 7 patients, and genome was detected in 12 patients. Panel B shows the results of RT-PCR of the CSF samples obtained from aseptic meningitis. Samples from B1 to B7 were obtained from the patients with aseptic meningitis during the course of natural mumps infection. Mumps virus was isolated from 1 patient and mumps genome was detected in 4 patients. For negative controls, we examined 1 CSF sample obtained from febile convulsion (lane B8) and 2 CSF samples obtained from Echo 30 aseptic meningitis (lanes B9 and B10), and mumps virus genome was not amplified.

Mumps virus genome was detected directly in NPS and CSF. Mumps virus was isolated in 28 (53.8%) of 52 NPS samples, while mumps virus genome was detected in 37 (71.2%) of 52 NPS samples, and mumps virus was isolated in 8 of 26 CSF samples (30.8%) while mumps virus genome was detected in 16 of 26 CSF samples (61.5%). The positive rate for the detection of mumps virus genome using RT-PCR was higher than that for mumps virus isolation in both clinical samples.

## Differentiation of Mumps Genome

The SH and HN regions were sequenced to demonstrate the longitudinal chronological genetic variability of mumps virus strains from 1976 in comparison with the Hoshino vaccine strain (data not shown). Sequence data provided an important information on genetic variability. Nucleotide difference between the Hoshino vaccine strain and wild mumps strains are shown in Figure 4. Nucleotides T at 1350 and G at 1656 were characteristic of the Hoshino vaccine strain, while all the wild strains were replaced to C at 1350 and A at 1656. Nucleotide position was numbered after intergenic sequence between the SH and HN genes. Two wild strains among more than 70 wild strains, which we sequenced so far, had nucleotide replacement from G to A at 1657, which were designed as the wild B type. The results of RFLP profiles of the Hoshino vaccine strains, the wild A strain, and the wild B strain, are shown in Figure 5. PCR product of the Hoshino vaccine strain was cut into two fragments, 470 bp and 71 bp with Sca I, and restricted into 2 fragments, 375 bp and 166 bp with Afl II. Wild A strains were not restricted with Afl II, but nucleotide replacement from G to A 1656 introduced a Sca I site at genome position from 1656 to 1661, resulting in two fragments of 382 bp and 159 bp after Sca I digestion. Only 2 wild strains (wild B) were cleaved neither with Sca I nor with Afl II. We examined 5 NPS samples obtained from the patients with acute parotitis and 14 CSF samples obtained from the patients with aseptic meningitis within 4 weeks after vaccination with the Hoshino vaccine strain. Some of the results of RFLP analysis were shown in Figure 6. We differentiated the vaccine strain from wild strains. The rate of mumps virus isolation, detection of mumps virus genome by RT-PCR, and detection of the Hoshino vaccine strain are shown in Table I. Mumps virus was isolated from 4 out of 5 NPS and 6 out of 14 CSF samples. RT-PCR increased the sensitivity of detection of the virus (11 of 14) from CSF samples. Two of 4 NPS samples and 9 of 11 CSF samples were identified as the Hoshino vaccine strain. Faint bands remained uncleaved at the original sizes in Figure 5 and Figure 6. These were not attributable to mixed population because these were all digested after increasing units of restriction enzymes.

PCR products were confirmed by direct sequencing.

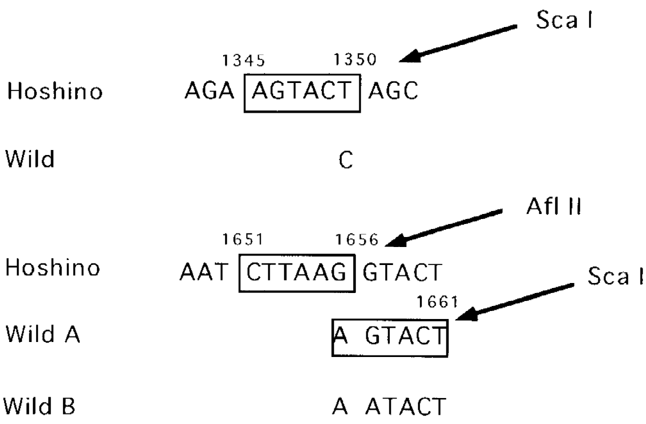


Fig. 4. Nucleotide differences of the amplified region between the Hoshino vaccine strain and wild strains.

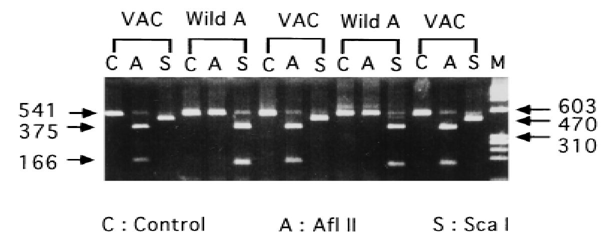


Fig. 6. RFLP analysis of PCR products amplified from clinical samples obtained from complicated cases after vaccination. RFLP profiles were shown in triplet of lanes. M, DNA marker.

## DISCUSSION

In April 1989, measles-mumps-rubella trivalent (MMR) vaccine, the AIK-C strain of measles vaccine, the Urabe Am9 strain of mumps vaccine, and the TO-336 strain of rubella vaccine was introduced into routine immunization program. An unexpected high incidence of aseptic meningitis was reported after MMR vaccination [Sugiura et al., 1991]. Yamada et al. [1990] reported the differentiation of the Urabe Am9 vaccine strain from wild strains by RFLP in part of the P gene, and later they described a more sensitive single-strand conformation polymorphism (SSCP) method [Katayama et al., 1995]. Most of the patients were identified with vaccine-related illness. There were several reports on the discriminination of vaccine strains from wild strains of mumps virus. Forsey et al. [1990] published the sequences of the gene coding for the fusion protein of mumps virus isolated from the patients with aseptic meningitis after Trivirix vaccination. Brown et al. [1991] described the sequence of the gene coding the haemagglutinin-neuraminidase (HN) gene of mumps virus isolated from patients with aseptic meningitis after Trivirix vaccination. Künkel et al. [1995] reported the differentiation of vaccine strain from wild mumps strains by using RT-PCR in the SH gene. They examined the clinical isolates from the patients and the results of discrimination depended upon the nucleotide sequencing. Yates et al. [1996] noted that different lineages of mumps virus were circulating and mumps vaccine strains, Urabe and Miyahara strains developed in Japan, were classified into different lineages from wild strains isolated in the United States and Europe. We sequenced a total of 2095 nucleotides of the SH and HN regions of 19 wild strains isolated from 1976 to 1994 in comparison with the Hoshino vaccine strain (unpublished data). Apart from these 19 wild strains, we sequenced the positive PCR products of the current wild strains detected by RT-PCR. All the wild strains had C at 1350, as did the Urabe and Miyahara vaccine strains. Two wild strains among more than 70 wild strains sequenced so far had nucleotide replacement from G to A at 1657, which were designated as wild B type. Nucleotides T at 1350 and G at 1656 were characteristic of the Hoshino vaccine strain, while all wild strains in Japan were replaced to C at 1350 and A at 1656.

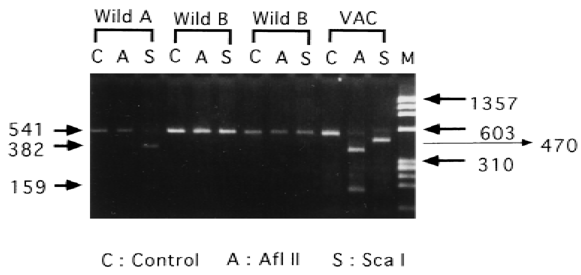


Fig. 5. RFLP analysis of PCR products of the Hoshino vaccine strain and wild strains. RFLP profiles of each strain were shown in triplet of lanes.

RFLP analysis was employed with Sca I and Afl II after the amplification in the part of the HN gene by RT-PCR directly from the clinical samples. Virus isolation is not efficient, since only 30–40% of clinical samples were positive. RT-PCR was developed for the detection of mumps virus genome directly from clinical samples. Cheek et al. [1995] reported that secondary vaccine failures were observed in the recipient of mumps vaccine. We examined 8 patients who had been immunized with mumps vaccine, and the mumps genome was detected in 7 of the 8 patients. Thus mumps virus infection can be identified more accurately by RT-PCR than by virus isolation. After discontinuation of further usage of MMR vaccine, monovalent mumps vaccines have been used in Japan. There are some complications after immunization with mumps vaccine. Parotitis was observed among 1% of the recipients of the Hoshino vaccine strain [Makino et al., 1990] and aseptic meningitis was reported at the rate of ap-

TABLE I. The Positive Rate of Mumps Virus Isolation and Detection of Mumps Virus Genome Through RT-PCR and Detection of the Hoshino Vaccine Strain

| | Positivity of virus isolation | Positivity of PCR | Incidence of vac. strain |
|---|---|---|---|
| NPS | 4/5 (80)[a] | 4/5 (80) | 2/4 (50) |
| CSF | 6/14 (42.9) | 11/14 (78.6) | 9/11 (81.8) |

[a]Percentages are given in parentheses.

proximately 1 case per 10,000 doses (data not shown). Because of the extremely low vaccine coverage, several outbreaks of mumps infection occurred in recent years. It is important to establish whether complications after vaccination are related to the vaccine strain or to the wild strain. A genetic differential method was applied to clinical samples obtained from the recipients with acute parotis swelling or aseptic meningitis within 4 weeks after vaccination. Wild virus strains were identified in some. All wild type strains were clearly different from the RFLP profile of the Hoshino vaccine strain. It is concluded that this molecular approach is a simple and reliable method to distinguish the Hoshino vaccine strain from wild strains.

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